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(54) Title: METHOD FOR THE GENERATION OF ANTIGEN-SPECIFIC T CELL LINES AND THERAPEUTIC USE THEREOF

(57) Abstract

A method is provided for the preparation of antigen-specific T helper or T cytotoxic cell lines which can be employed as therapeutic agents to increase the mammalian immune response to a pathogen, such as a virus, which incorporates or causes the cellular expression of said antigen.

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METHOD FOR THE GENERATION OF ANTIGEN-SPECIFIC T CELL LINES AND THERAPEUTIC USE THEREOF

Cross-Reference to Related Application

This application is a Continuation-in-Part of U.S. application Serial No. 933,789, filed November 24, 1986.

Background of the Invention

The invention described herein was made with the assistance of National Institutes of Health Grant No. 1-P01-HD19937-01A1. The Government has certain rights in this invention.

Although the mechanisms and agents involved in the mammalian immune response reaction are not fully understood, techniques to manipulate the immunologic response have great therapeutic potential. This is especially apparent in the care of pathological conditions which result in suppression of the normal immune responses and which are not readily amenable to treatment by conventional drug-based therapies. Such conditions include certain viral infections, and the immunosuppression caused by the administration of anti-cancer drugs, drugs employed to treat autoimmune diseases, and those used to inhibit organ rejection following transplantation.

The capacity to respond to immunologic stimuli rests primarily in the cells of the lymphoid system. During embryonic life, a stem cell develops, which differentiates along several different lines. For example, the stem cell may turn into a lymphoid stem cell which may differentiate to form at least two distinct lymphocyte populations. One population, called T lymphocytes, is the effector agent in cell-mediated immunity, while the other (B lymphocytes) is

the primary effector of antibody-controlled, or humoral immunity. The stimulus for B cell antibody production is the attachment of an antigen (Ag) to B-cell surface immunoglobulin. Thus, B cell populations are largely responsible for specific antibody (Ab) production in the host. At times, and for certain Ags, B cells require the cooperation of T cells for effective Ab production.

Of the classes of T lymphocytes, T helper (T_h) cells are antigen-specific cells that are involved in primary immune recognition and host defense reactions against bacterial, viral, fungal and other antigens. The T cytotoxic (T_c) cells are antigen-specific effector cells which can kill target cells following their infection by pathological agents.

While T helper (T_h) cells are antigen-specific, they cannot recognize free antigen. For recognition and subsequent T_h cell activation and proliferation to occur, the antigen must be presented to receptors or a receptor complex on the T_h cell together with major histocompatibility complex (MHC) class II products, or "leukocyte antigens". Thus, T_h cell recognition of pathogenic antigens is MHC class II "restricted" in that a given population of T_h cells must be either autologous or share one or more of the restricting leukocyte antigen (LA) specificities expressed by the MHC of the host. Likewise, T_c cells recognize Ag in association with class I MHC LAs.

In the case of T_h cells, this function is performed by a limited number of specialized cells termed "antigen-presenting cells" (APC). It is now well-established that T helper (T_h) cells recognize processed soluble antigen in association with class II MHC LA, expressed on the surface of macrophages. Recently, other cell types, such as resting and activated B

cells, dendritic cells, epidermal Langerhan's cells and human dermal fibroblasts, have also been shown to present antigen to T cells. Epstein-Barr virus transformed human lymphoblastoid B cells (LCL) have been shown to present tetanus toxoid, M. Leprae and Candida albicans to autologous antigen-specific T cells.

If a given T_h cell possesses receptors or a receptor complex which enable it to recognize the MHC-class II LA-antigen complex, it becomes activated, proliferates and generates lymphokines such as interleukin 2 (IL-2). The lymphokines in turn cause the proliferation of several types of "killer cells", including T_c cells and macrophages, which can exhibit antimicrobial and tumoricidal activity. After stimulation subsides, survivors of the expanded T_h cells remain as memory cells in the body, and can expand rapidly again when the same antigen is presented. The importance of T cells in the recovery from acute viral infections has been well established for some viruses, in particular the myxovirus, (influenza), the poxviruses (ectromelia and vaccinia), and the arenavirus, (lymphocytic choriomeningitis virus).

Despite the well-established importance of the defense mechanisms of the immune system to the well-being of the host, the therapeutic potential of these agents has not been realized. In a preliminary study, S. A. Rosenberg et al., New England J. Med., 313, 1485 (1985) reported that the systemic administration of autologous peripheral blood lymphocytes (PBL) which had been incubated with IL-2, along with additional IL-2, to patients with advanced cancer achieved cancer regression in 11 of the 25 patients treated. Rosenberg et al. termed the incubated PBLs "lymphokine-activated killer (LAK) cells" and reported that they were members of a cytolytic system which is distinct from that of

natural killer cells and cytotoxic T cells. However, subsequent studies employing this therapy have not confirmed the promise of these early results.

Whatever the long-term results of this approach to "adoptive immunotherapy", Rosenberg et al. noted that "[t]he major difficulty in the application of this approach to the treatment of human cancer has been the inability to generate sufficient numbers of autologous human cells with antitumor reactivity that could be used for systemic therapy." This is particularly problematic in the case of patients who are immunosuppressed due to infections, cancers, organ transplant or anti-neoplastic drugs and the like.

Likewise, numerous attempts have been made to isolate and maintain homogeneous populations of T_c or T_h cells and to characterize them in terms of their antigen specificity and MHC restriction. These attempts usually involve the stimulation of mononuclear cells from a seropositive human or murine host with bacterial or viral preparations in combination with non-proliferative APCs, such as irradiated autologous mononuclear cells (MNCs). Proliferating polyclonal populations of T_h cells or T_c cells are cloned by limiting dilution to obtain homogeneous populations and then further proliferated and characterized by a variety of techniques. As noted by Rosenberg et al. in the case of cloned LAK cells, one of the major obstacles in cloning T lymphocytes is the limited availability of autologous, or alternatively, allogeneic MHC LA-matched MNCs, especially from clinical subjects.

To overcome this problem, APCs other than autologous MNCs have been employed as APCs. For example, T. Issekutz et al., J. Immunol., 129, 1446 (1982) first disclosed that autologous Epstein-Barr

virus (EBV)-transformed LCL lines can present antigens associated with tetanus toxoid to tetanus-reactive polyclonal T cells and T cell clones. D. R. Kaplan et al., in Cellular Immunology, 88, 193 (1984) reported the production of three T_c cell clones by the proliferation of peripheral blood MNCs from a type A influenza immune donor. One of the clones proliferated in the presence of irradiated, virus-infected, autologous MNCs or in the presence of irradiated, infected Epstein-Barr virus transformed allogeneic lymphoblastoid cells (LCL).

B. G. Elferink et al., Scand J. Immunol., 22, 585 (1985) further showed that autologous and allogeneic Epstein-Barr Virus (EBV)-transformed LCL lines can present antigens associated with M. leprae bacili to M. leprae-reactive cloned T cell lines. A further paper by this group described the isolation of a T cell clone which may recognize only M. leprae antigens. The cloning method used autologous EBV-transformed LCLs as APCs. The advantage of using EBV-transformed LCLs is that they may provide a continuous and unlimited source of APCs. [J.B.A.G. Haanen et al., Scand. J. Immunol., 23, 101 (1986).]

Human cytomegalovirus (HCMV) is a large species-specific herpesvirus (DNA $1.5X10^8$ Da) which shares the properties of latency and reactivation with other members of this group. HCMV is ubiquitous (30-70% of adults are seropositive) but the principal site(s) of latency of HCMV are uncertain, as are the molecular events involved in its reactivation. HCMV infection and reactivation can be asymptomatic, but are associated with appreciable morbidity and mortality in immunosuppressed subjects. For example, HCMV is the most common cause of opportunistic infection in bone marrow transplant patients. More than 80% of those who

develop HCMV pneumonia die despite current treatment modalities.

Whereas monoclonal antibody therapy may be helpful as a prophylactic treatment, it has been suggested that survival of immunosuppressed patients with serious HCMV infections may be dependent on the presence of HCMV-specific T cytotoxic cells. Quinnan et al., in New England J. Med., 307, 7 (1982) have suggested that HCMV-specific cytotoxic T cell responses in these patients are associated with clinical recovery and cessation of viral excretion, whereas those with active infections who did not develop cytotoxic responses uniformly died.

R. C. Gehrz et al., Lancet, 2, 844 (1977), disclosed that infants with congenital HCMV infection have an antigen-specific defect in T helper cell proliferation which is associated with persistence of replicating viral infection for months to years despite the presence of HCMV-specific antibodies. Acquisition of HCMV-specific lymphocyte proliferative responses appears to be associated with a diminution in viral excretion. Thus, it is likely that HCMV-specific T helper cells play a significant role in immune defense against this virus in immunocompetent hosts.

In patients with reactivated HCMV infection, antibodies directed against HCMV antigens are not protective or are only useful in conjunction with appropriate T cell responses. Peripheral blood lymphocytes (PBL) are also not likely to be useful as therapeutic agents, even if they could be obtained in sufficient quantities. The numbers of T helper cells or T cytotoxic cells reactive with a particular antigen are extremely low and thus, selective expansion of antigen-specific T cells in vitro will be required to obtain sufficient numbers for therapeutic purposes.

Moreover, therapeutic administration of allogeneic PBLs would activate T cells recognizing "foreign" leukocyte antigens, resulting in a mixed leukocyte culture reaction. This mixed leukocyte culture reaction may activate undesirable non-specific immune responses, or alternatively, induce suppressor cells which might inhibit desired antigen-specific immune responses.

Autologous or allogeneic antigen-specific T cell lines are likely to express a desired therapeutic activity without attendant complications associated with the mixed leukocyte culture reaction. L. K. Borysiewicz et al., Eur. J. Immunology, 13, 804 (1983) reported the generation of short-term polyclonal T_h cell lines by the expansion of MNCs from seropositive subjects with soluble HCMV antigen in the presence of IL-2. When the MNCs were co-cultured on autologous HCMV-infected fibroblasts, polyclonal T_c cells were generated, which lysed HCMV-infected cells.

However, a need exists for improved methods to generate and maintain populations of T_h cells and T_c cells which are specific to antigens associated with viruses such as HCMV and other pathogenic agents. A further need exists for immunotherapeutic methods based upon the administration of antigen-specific T lymphocyte populations of known biological activity to mammalian subjects.

Brief Description of the Invention

I. Production of T Cell Lines

A. T Helper Cells

The present invention is directed to methods for the efficient production of a homogeneous population of T helper (T_h) cells which are specific for a viral antigen, such as an HCMV antigen. It is believed that the present methods provide the basis for the

efficient production of cloned T_h cells while using a minimum amount of antigen and lymphokine support, while maintaining both the antigenic specificity and functional activity of the clones. In its broadest aspect, the present method comprises:

(a) isolating a population of mononuclear cells (MNC) from the blood of a donor mammal, wherein the MNC population comprises T helper cells specific for a viral antigen, and autologous, non-proliferative, antigen-presenting cells (APC);

(b) combining said isolated population of said MNCs with an amount of the viral antigen effective to cause the proliferation of a T helper cell which is specific for the antigen;

(c) allowing said T helper cell to proliferate for a period of time effective to allow non-proliferating MNCs to lose viability; and

(d) clonally expanding the antigen-specific T helper cell in the presence of an amount of non-proliferative antigen-presenting cells comprising a mixture of (i) autologous MNCs, allogeneic MNCs or mixtures thereof; and (ii) autologous lymphoblastoid cells (LCLs), allogeneic LCLs or mixtures thereof; and an amount of said viral antigen effective to proliferate said cloned antigen-specific T helper cell, to yield said homogeneous population, and wherein said LCLs are present in an amount effective to increase the proliferation rate of the T helper cell over that caused by the MNCs.

During the selective proliferation step (c), which may require 1-2 weeks, it can also be effective to add an additional amount of said viral antigen and the autologous, non-proliferative APCs, in order to cause the further proliferation of the T helper cell, so as to enhance the production of a viable polyclonal population of viral antigen-specific T_h cells prior to step (d). In step (d), antigen-specific monoclonal T_h cells may be derived by limiting dilution, or by single cell isolation by micromanipulation or flow cytometry.

Antigen-specific polyclonal T cell lines, as well as monoclonal antigen-specific T cells, have potential therapeutic benefit. Thus, the term "T cell line" as used herein may appropriately refer to an expanded population of polyclonal T cells which is uniquely reactive with a given antigen, or to a homogeneous population of monoclonal T cells which has been derived by the expansion from a single progenitor T cell.

Since the present invention comprises the proliferation of antigen-specific T_h cells, it also provides a method for proliferating a homogeneous population of T helper cells which are specific for a viral antigen comprising combining said homogeneous population of T helper cells with an amount of the antigen and an amount of a mixture of non-proliferative, antigen-presenting cells (APC) effective to cause the proliferation of the population of T helper cells, wherein said mixture of APCs comprise (i) autologous or allogeneic lymphoblastoid cells (LCL) and (ii) autologous or allogeneic mononuclear cells (MNC), wherein the LCLs are present in an amount effective to increase the proliferation rate of the T helper cells over that caused by the MNCs. Preferably, the LCLs are derived from a continuous lymphoblastoid cell line, such as can

be produced by viral transformation, hybridoma technology and the like. The APCs are rendered non-proliferative by techniques known to the art, e.g., by x-irradiation, treatment with chemical agents such as mitomycin C and the like.

Both non-proliferative MNCs and LCLs have been found to cause the proliferation of T_h cells when they are derived from the same host as the T_h cells or are allogeneic MNCs or LCLs which share one or more class II restricting antigens (abbreviation: "MHC LA-matched"). It was surprisingly found that a mixture of LCLs and MNCs can synergistically present antigen to T_h cells, thus substantially increasing their proliferation rate over that caused by an equivalent number of either type of APC when used alone.

For example, this effect is observed in the case of a viral antigen such as an HCMV antigen, when a mixture of EBV-transformed autologous LCLs and autologous MNCs are employed as the APCs in a ratio of at least about 1:10. Preferably, the ratio of T_h cells to LCLs added is about 1:1-3 and the ratio of T_h cells to MNCs is about 1:3-10.

Furthermore, only a limited density of LCL is required to substantially augment the expansion of T_h clones. This aspect of the present method is important from a practical point of view in that human antigen-specific T_h clones can be expanded into large quantities in a relatively short period of time using limited numbers of MNCs. For example, in accord with the present method, HCMV-specific T_h clones could be expanded from $1-2\times10^6$ cells to 10^9 cells in 2 weeks by using $1-2\times10^7$ MNC and 1×10^6 LCL as APC.

It has also been found highly preferable to carry out proliferation steps (c) and/or (d) of the present method in the presence of an effective amount of interleukin-2 ("IL-2" or "TCGF").

A further aspect of the present invention comprises increasing the proliferation rate of the viral antigen-specific T_h cells by conducting the initial proliferation step (steps a-c, hereinabove) or the subsequent expansion step (step (d), hereinabove) in the presence of an amount of monoclonal antibody which is specific for said viral antigen. In one embodiment of this aspect of the invention, the proliferation rate of a T_h cell specific for an HCMV viral antigen is increased by combining MNCs isolated from the blood of an HCMV-seropositive donor mammal with a monoclonal antibody specific for an antigen present on an HCMV gene product such as a structural protein. The monoclonal antibody is employed in combination with (a) an effective proliferation-stimulating amount of said antigen, and (b) an amount of autologous APCs selected from the group consisting of (i) non-proliferating MNCs, (ii) a non-proliferating LCL derived from a continuous LCL, and (iii) mixtures thereof, e.g., mixtures which can cooperatively interact to further increase the proliferation rate of the T_h cells. Preferably, the autologous, non-proliferating antigen-presenting cells are selected from the group consisting of x-irradiated autologous MNCs, an x-irradiated Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell line (LCL) and mixtures thereof. These are the preferred APCs for use in step (b) of the present method, whether or not monoclonal antibody and/or IL-2 is employed to increase the proliferation rate.

8. T Cytotoxic Cells

The present method can also be employed to produce a homogeneous population of T cytotoxic (T_c) cells which are specific for an HCMV antigen. This aspect of the present invention comprises the steps of:

(a^1) isolating a population of mononuclear cells (MNC) from the blood of a donor mammal, preferably a human, wherein the MNC population comprises T_c cells specific for said viral antigen;

(b^1) combining said isolated population of said MNCs with (i) an amount of autologous, non-proliferative, antigen-presenting cells (APC), e.g., irradiated MNCs, (ii) an amount of said HCMV antigen, and (iii) an amount of interleukin-2 (IL-2), effective to cause the proliferation of a T_c cell which is specific for said antigen;

(c^1) allowing said T_c cell to proliferate for a period of time effective to allow non-proliferating MNCs to lose viability; and

(d^1) clonally-expanding said antigen-specific T_c cell in the presence of (i) an amount of non-proliferative, autologous, antigen-presenting cells; non-proliferative, allogeneic, antigen-presenting cells or mixtures thereof, (ii) an amount of IL-2 and (iii) an amount of said HCMV antigen, effective to proliferate said cloned antigen-specific T_c cell, to yield said homogeneous population.

Steps (b^1), (c^1) or (c^1) are preferably carried out in the presence of a monoclonal antibody which is specific for an HCMV antigen, and which acts to increase the proliferation rate of said T_c cell.

As previously described for antigen-specific T helper cell lines, polyclonal T_c lines reactive with a

particular viral antigen can be expanded by continuous stimulation of T_C blasts exhibiting cytotoxic activity against target cells expressing the desired antigen. Homogeneous populations of virus-specific T_C clones derived from a single progeny T cell may be obtained by limiting dilution, or by single cell isolation by micromanipulation or by flow cytometry, followed by expansion according to step (d^1).

Other preferred embodiments of this T_C cell proliferation method include (1) the use of whole HCMV-viral antigen or the use of HCMV-infected autologous or allogeneic fibroblasts as a source of cell-associated viral antigen, e.g., an HCMV immediate-early protein; (2) the addition of additional amounts of the viral antigen, IL-2 and the autologous, non-proliferative APCs during the course of step (c^1) to cause further proliferation of said T_C cell, and (3) the use, in step (b^1) or in step (d^1), of antigen-presenting cells which further comprise non-proliferative MNCs derived from a continuous autologous MNC line in an amount effective to increase the proliferation rate of the T_C cells over that caused by the autologous mononuclear antigen-presenting cells. This continuous, autologous MNC line preferably comprises a virus-transformed LCL, i.e., an EBV-transformed LCL.

The present invention is also directed to a method for proliferating a homogeneous population of T_C cells which are specific for an antigen, such as a viral antigen, i.e., an HCMV antigen. The method comprises combining said homogeneous population of T cytotoxic cells with an amount of said antigen, an amount of interleukin-2 (IL-2) and an amount of a mixture of non-proliferative, antigen-presenting cells (APC) effective to cause the proliferation of said population of T_C cells, wherein said mixture of APCs

comprise allogeneic virus-transformed lymphoblastoid cells (LCL) and allogeneic mononuclear cells (MNC). The method further comprises combining said population of T_c cells with an amount of monoclonal antibody specific for said antigen, which monoclonal antibody acts to increase the proliferation rate of said population of cells.

As used herein with respect to a pathological target such as a virus or an infected cell, the term "antigen" refers to a compound such as a polypeptide, polypeptide complex, glycoprotein, nucleic acid or the like, which elicits an immune response. A pathological target antigen may be a portion of the pathological target itself, e.g., a viral envelope glycoprotein, or it may be an antigen expressed by a diseased tissue such as neoplastic tissue or a virus-infected cell. An "antigen-specific" T lymphocyte becomes activated in the presence of a single antigen when it is presented by an antigen-presenting cell (APC), in association with a specific leukocyte antigen (LA) expressed by the antigen-presenting cell. An antigen-specific T_h cell will proliferate in a suitable medium in the presence of the antigen for which it has specificity when said antigen is presented to the T_h cell by an APC which also expresses the leukocyte antigen for which the T_h cell has specificity. As noted hereinabove, in the case of human T helper cells, the specific leukocyte antigen will be a human MHC class II antigen, whereas human T cytotoxic cells are specific for MHC class I antigens.

C. HCMV Antigen-Specific T Cell Lines

Preferred embodiments of the present invention comprise homogeneous populations of T_c or T_h cells, and methods for the production thereof, wherein a given cell population is specific for an HCMV antigen.

When a T cell or a T cell clone is said to be antigen-specific, although the composition comprising the antigen may be specified, the epitope, or specific antigenic site on the antigenic composition, is not necessarily known. For example, three different homogeneous populations of T helper lymphocytes may be specific for one particular HCMV structural protein or glycoprotein. While they all respond to the same antigen, and thus, have the same antigen specificity, they may have different specificities at the submolecular level, meaning they may respond to different regions of the antigen, or epitopes. Within this context, different HCMV-specific T lymphocytes have the same submolecular-specificity only if they recognize the same epitope on the same HCMV-associated antigen. They may still be specific for the same antigen, however, if the antigen has a plurality of epitopes as presented by APCs in different instances.

For example, the HCMV DNA genome is transcribed in sequential order, beginning with the restricted transcription of immediate-early genes encoding regulatory proteins required for subsequent expression of early genes. The major immediate-early gene (I-E1) encodes a 68 kD regulatory protein which is expressed on the membrane of infected cells 6-24 hours after onset of infection. HCMV-specific T cytotoxic cells are thought to primarily recognize this immediate-early protein as part of their role in immune surveillance to prevent reactivation of latent HCMV.

Transcription of early genes precedes the onset of DNA synthesis. Included among the early gene products are virus-specific polymerases and kinases necessary for DNA replication. These enzymes do not appear to play an important role in immune responses.

However, the late HCMV genes encode a variety of immunogenic structural proteins and glycoproteins.

Included among these proteins are disulfide-bridged envelope glycopeptide complexes, non-glycosylated envelope proteins, tegument glycoproteins bridging the viral nucleocapsid with the outer envelope; and matrix proteins forming the internal capsid structure of the virus.

The purification and characterization of a number of immunogenic structural glycopeptide complexes and reduced glycopeptides from the HCMV envelope fraction, along with monoclonal antibodies (MoAbs) specific thereto, is fully disclosed in U.S. application Serial No. 933,789, filed November 24, 1986. the disclosure of which is incorporated by reference herein. The primary glycopeptides, glycopeptide complexes and MoAbs described therein are summarized in Table 1, below.

TABLE 1

HCMV Surface Glycopeptide Complexes and Glycopeptides Immunoprecipitated by MoAbs

Ion-Exchange HPLC Peak* (MoAb Reactivity)	Glycopeptide Complex Immunoprecipitated (mol wt., kD)	Glycopeptides Immunoprecipitated After Reduction (mol wt., kD)
2 (9E10)+	93	50-52**
4 (9E10)	450	50-52**, 90, 116, 130, >200
3 (41C2, 987)	130, and >130	50-52, 93++, 130
2 (none)	93 kD Glycopeptide not associated in Disulfide-linked Complex	93 kD Glycopeptide*** not Immunoprecipitated by any MoAb

* The Detergent Extraction, HPLC, Reduction, Immunoprecipitation and Electrophoresis Methodology employed to obtain these materials from a purified whole virus preparation (designated "Whole HCMV Antigen" in Example I, below) is fully set forth in B. Kari et al., J. Virology, 60, 345 (1986), the disclosure of which is incorporated by reference herein.

+ The hybridomas producing these MoAbs have been deposited with In Vitro International, Linthicum, MD, and have been assigned the following access codes: Hb 2-29-9B7 ("9B7") = IVI-10117; Hb 2-15-9E10 ("9E10") = IVI-10118; Hb 1-48-41C2 ("41C2") = IVI-10119.

++gpA, **gpB, ***gpC.

The immunogenic glycopeptide complexes and glycopeptides listed on Table 1 are useful to selectively stimulate the proliferation of T_h cells derived from the blood of an HCMV-seropositive donor. Likewise, monoclonal antibodies of known binding specificity such as 9E10, 41C2 and 9B7 can be used to purify viral antigens. These monoclonal antibodies can also be used to augment antigen recognition by T_h cell clones, thereby facilitating their expansion in the presence of limited amounts of viral antigen.

II. Immunotherapy with T Cell Lines

T cell recognition of different HCMV proteins is important in planning the proper course of immunotherapy with the T cell lines. The T_h cells exemplified herein primarily recognize structural proteins of HCMV. They are, therefore, likely to be important in the recognition of cell-free virus (i.e., in cases of acute viremia). Data in mice and humans show that certain T cytotoxic cells recognize immediate-early proteins, which are expressed on the surface of infected cells. Since these proteins are only produced after the viral genes are expressed by the host cells, such T_c cells are important in preventing reactivation of latent HCMV and their administration may be indicated for both prophylaxis and specific HCMV treatment.

The preferred T cell lines recognize viral antigens in association with human LA products. Therefore, therapeutic T cells must be either autologous or share one or more of the restricting specificities expressed by the patient. In the context of the transfer of isolated T cells to an individual as a means of therapy, allogeneic cells are cells from a donor individual of the same species as the recipient which are major histocompatibility complex matched for an appropriate human leukocyte antigen (HLA) class of antigens expressed by the recipient (abbreviation "MHC LA-matched"). Therefore, when dealing with a patient with a pathological infection such as chronic HCMV disease, one can expand autologous antigen-specific T cell lines in vitro for subsequent T cell therapy via re-administration. Alternatively, when rapid access to T cell clone reagents is important to treat acute life-threatening infection, pre-expansion and administration of a purality of expanded allogeneic therapeutic T cells of known viral antigen-specificity and/or HLA type can be employed. This technique is particularly important in the case of a patient who is immuno-suppressed.

Therefore, a further aspect of the present invention is directed to a method of using T lymphocytes (T cells) for a therapeutic treatment of a mammal, such as a human patient, having a viral infection, such as an HCMV infection. The method comprises treating said infected mammal with an amount of a homogeneous, clonally-expanded, viral antigen-specific T cell population effective to elicit an increased immune reaction to the viral infection, wherein said T cell population is specific (a) for at least one leukocyte antigen (LA) present on the surface of an antigen-presenting cell (APC) of said mammal, and (b) for an antigen of said virus.

Preferably, the clonally-expanded T cell population will consist essentially of T_h cells or T_c cells. As discussed hereinabove, it is often preferable to administer a plurality (or "bank") of homogeneous, clonally-expanded, viral antigen-specific T cell populations in order to (a) promote immune system recognition of antigens expressed during different stages of infection and/or (b) to guarantee that an effective number of the total T cell populations will be MHC-matched to the recipient.

Therefore, the present method of T cell immunotherapy comprises the administration of a plurality of T cell populations which comprise T cells having specificity (a) for a plurality of different antigens of said virus, and (b) for at least one LA present on the surface of an APC of said mammal. Furthermore, the present method can also comprise the administration of a plurality of T cell populations, each comprising T cells having specificity (a) for at least one antigen of said virus, and (b) for a plurality of different LAs present on the surfaces of APCs of a plurality of allotypes of a single species of said mammal, wherein at least one LA is LA-restriction-matched with said mammal.

Polyclonal lines as well as T cell clones may have significant therapeutic potential. A particular advantage of antigen-specific polyclonal T cell lines is that they include T_h and/or T_c cells which can recognize the target viral antigen in association with all restricting MHC LAs expressed by the T cell donor. The advantage of virus-specific T_h and T_c clones derived from a single progenitor T cell is that they represent a homogeneous population with cells of known antigen specificity, functional activity, and MHC LA-restriction specificity. Therefore, they are likely to

exhibit maximal therapeutic effect since all cells in the population exhibit the same functional activity. From a practical point of view, however, a much larger "bank" derived from discrete T cell populations will be required if monoclonal T cells are to be used.

Since the methods of the present invention permit the administration of a therapeutically-effective amount of T_h cells and/or T_c cells, the afflicted mammal may, but need not be, concurrently treated with exogenous lymphokines such as IL-2 (TCGF). Since the administration of large doses of IL-2 has been associated with adverse reactions in some patients, the ability to enhance the immune response in the absence of IL-2 can provide a substantial improvement in the efficacy of T cell therapy.

Representative antigen-specific T cell lines which have been generated in accord with the present methods and which are representative of the populations which can be used to generate the T cell "banks" discussed above are listed in Table 2, below.

Table 2: T Cell Lines

T Cell	Designation	HCMV Antigen Specificity	IVI Access[d] Code
T_h[a]	WRC-T3#3 (SP-CN/LCL)	gpA	10124
T_h[a]	WRC-T2#41	gpA	n/a
T_h[a]	WRC-T2#88	64 kD matrix protein	10125
T_h[a]	WRC-T2#131	64 kD matrix protein	n/a
T_c[b]	SP-CN CA-1	I-E1[c]	10126
T_c[a]	SP-RK-3	structural protein	10127

[a] clonal [b] polyclonal [c] Immediate-Early Protein (68 kD)

[d] Deposited with In Vitro International, Lithicum, MD, in accord with the Draft Patent and Trademark Office Deposit Policy for Biological Materials, BNA PCTJ, 32, 90 (1986).

The present invention has been described primarily in terms of the production of T_h and T_c cells which are specific for viral antigens, and the treatment of viral infections with homogeneous populations of these T cells. However, it is believed that homogeneous populations of T_h and T_c cells which are specific for antigens on a wide variety of pathological targets can be prepared using the present method, and that these populations will be effective to augment or stimulate the immune response to a wide variety of pathogens or pathological targets .

Defined broadly, a pathological target is an entity within a mammalian body which is the cause of, or a result of, a disease which threatens the health and well-being of the mammalian host. The target may

be an entity which is foreign to the host such as a virus, bacterium, fungus, or the like, or it may be a product of a disease or pathological condition, such as a virus-infected cell, a neoplastic cell, and the like. In specific embodiments of the therapeutic treatment of the present invention, the pathological target of the treatment is occasionally a secondary infection which is more threatening to the intended recipient because a primary illness has weakened the recipient's ability to respond immunologically to the secondary infection. Many aspects of the primary illness may weaken the intended recipient's immune response to the secondary infection, including therapeutic treatments which act to suppress aspects of the recipient's immune system. Such treatments include, but are not limited to, radiation therapy, chemotherapy and the like.

In summary, the present therapeutic method can employ allogeneic T cells from individuals of the same species who are MHC LA-matched. This will enable physicians and pharmacists to simply use pre-formed dosage forms of allogeneic T cells to treat a patient. Since T cells which are not matched are simply ineffective in aiding the patient, the dose may contain many cells, some of which are matched and will aid the patient and some of which are not matched and will not aid the patient.

In this way, a single dosage form may be used to treat a number of patients sharing one or more LAs expressed by the allogeneic therapeutic cells. In addition, the dose may contain both T_c and T_h cells which have different MHC class restrictions. Obviously, such a therapeutic treatment does not require the preparation of doses on a case-by-case basis. This means that the T cells can be expanded in large culture mediums, allowing for efficiencies of

scale. In addition, the cells can be made immediately available without requiring individualized collection, leukapheresis and culture.

Notwithstanding the foregoing advantages, however, the most promising feature of this new immunotherapy is the prospect that each dose will contain antigen-specific T cells. This represents a substantial advance over the Rosenberg et al. method which affords only non-specific enhancement of cytotoxic cells. In the present method, a dosage form may be provided which contains allogeneic T_h and T_c cells which are specific for a variety of antigens which are all associated with a particular pathogen. For instance, a unit dosage may contain a plurality of T_h and T_c cell clones which have been individually cultured and then mixed. The dosage form may contain T_h and T_c cells with a pre-selected number of LA restrictions, and also a pre-selected number of antigen specificities. If a particular pathogen is known to express 10 or 20 known epitopes on a variety of antigens, structural or otherwise, the dose may contain appropriate T cells having specificity for each.

Treatment methodologies will normally involve the parenteral administration of a therapeutically effective amount of autologous or allogeneic T cells in a suitable liquid vehicle, such as a physiological salt solution. One such protocol is that provided by S. A. Rosenberg et al., New England J. Med., 313, 1485 (1985), but the number of cells delivered per dose, and the number of doses administered will vary widely, depending upon factors determined by the clinician, including the pathology under treatment, the physique and the physical condition of the patient and other factors. However, due to the enhanced efficiency of the proliferation method of the present invention, it

may be possible to remove substantially fewer MNCs from the patient, and to return substantially more autologous antigen-specific T cells to the patient, than called for by the Rosenberg et al. methodology.

Detailed Description of the Invention

The invention will be further described in accord with the following detailed examples.

EXAMPLE I

1. Preparation of Viral Antigens

Human primary fibroblast cultures grown in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum were infected with Towne strain HCMV at an multiplicity of infection (MOI) of 1-5. At 3-4 days post infection [^{3}H]glucosamine (5 uCi/ml, 22 Ci/mM, Amersham, Arlington Heights, IL) or [^{35}S]methionine (5 uCi/ml, 109 Ci/mM, DuPont/NEN, Boston, MA) was added as a marker during subsequent purification, for certain of the experiments described hereinbelow.

Cells and cellular debris were removed from the medium by low-speed centrifugation at 7500 x g for 20 min. Virus was collected from the supernatant by centrifugation at 48,200 x g for 1 hr. The viral pellet was resuspended in Tris NaCl buffer (50 mM trishydroxymethylamino methane-HCl, pH 7.4, and 150 mM NaCl) and layered onto 20-60% sucrose gradients made with the same buffer. Velocity sedimentation was done at 131,300 x g for 1 hr. Gradients were collected and monitored for optical density at 280 nm. A major peak of optical density which banded at 43% sucrose was collected. These fractions were diluted with Tris NaCl buffer and virus collected by centrifugation at 131,300 x

g for 2 hrs. This purified whole virus preparation was designated "Whole HCMV Antigen".

In order to solubilize membrane constituents, the whole HCMV antigen preparation was resuspended in 2-4 ml TN buffer (Tris-NaCl buffer (50 mM Tris hydrochloride, pH 7.5, 10 mM NaCl, 2 mM phenylmethylsulfonyl fluoride)) containing 1% Triton-X 100 (TX-100). After incubation at ambient temperature for 60 minutes, this solution was layered onto a linear 20-60% sucrose gradient for rate zonal centrifugation at 120,000 g for 60 minutes. The TX-100 solubilized material remained at the top of the gradient, while the HCMV nucleocapsids were banded by velocity sedimentation. Alternatively, detergent extracts of Towne strain HCMV were prepared using 1.0% Nonidet P-40 (NP-40, Sigma Chemical Company). In this method, 10-15 mg of the purified whole HCMV antigen were suspended in 3-5 ml of TN buffer containing 1% NP-40. Detergent-virus suspensions were stirred for one hour at room temperature. The extracted proteins were separated from insoluble proteins by high-speed centrifugation for 1 hour. The extract obtained by either of these methods was designated "HCMV detergent extract".

Whereas HCMV-specific T helper cells appear to recognize HCMV proteins and glycoproteins contained within the whole HCMV antigen preparations, HCMV-specific cytotoxic T cells recognize primarily HCMV-encoded proteins expressed on the surface of infected cells. In particular, the major immediate-early (I-E1) protein, which is expressed on the cell membrane within 6-24 hours of infection, appears to be important in expansion of HCMV-specific cytotoxic T cells.

Therefore, autologous fibroblasts or allogeneic fibroblasts sharing one or more class I leukocyte antigens recognized by the T cell donor were infected with Towne strain HCMV at a multiplicity of infection of 5-10 for 3 hours. In some cases, the cells were infected in the presence of cyclohexamide (50 µg/ml). After removal of cyclohexamide, Actinomycin D (5 µg/ml) was added to prevent further DNA transcription, thereby allowing for selective expression of I-E genes of HCMV. Infected fibroblasts not treated with cyclohexamide and Actinomycin D expressed both I-E and late gene products of HCMV. These preparations were designated "cell-associated viral antigen".

Herpes simplex virus type I (HSV-I) was partially purified as described by R. C. Gehrz et al., Lancet, 2, 844 (1977) and heat-inactivated at 56°C for 1 hr. to yield "HSV Antigen".

EXAMPLE II

1. Generation of HCMV-Soecific T Helper Cell and T Cytotoxic Cell Lines

HCMV-specific T cell blasts were prepared in 25 cm^2 upright tissue culture flasks by stimulating 10 x 10^6 mononuclear cells (MNC) at a concentration of 1 x 10^6 MNC/ml with 10 µg of heat-inactivated whole HCMV antigen (56°C, 1 hr) suspended in RPMI 1640 medium supplemented with 15% heat-inactivated HCMV-seronegative human serum (PHS). After 7-10 days at 37°C in 5% CO_2 atmosphere, the HCMV-specific blasts were either further expanded in bulk culture, or cloned by limiting dilution in 96 well U-bottomed tissue culture plates at 0.3 cell/well in the presence of x-irradiated (5,000 R) autologous MNC (MNC^{IRR}) as feeder cells, whole HCMV antigen at a

concentration of 1 µg/ml (for T_h cells) or cell-associated antigen at a fibroblast:MNC ratio of 1:20 (for T_c cells), and 10-20% interleukin-2 (IL-2) (TCGF, Biotest, Frankfurt, Germany). Thereafter, cells were refed every 3-4 days with fresh IL-2-containing media. Whole HCMV antigen and autologous x-irradiated feeder cells were added to the media every 7-14 days.

Growing cells were fed and transferred to 96 well flat-bottomed tissue culture plates. Growing clones were then transferred to 24 well plates for further expansion and then reseeded into 25 cm^2 tissue culture flasks for large scale production. Expanded clones were subcloned by a second limiting dilution to ensure clonality. Clones were developed from 4 different HCMV-seropositive donors yielding more than 100 individual T cell lines.

2. Characterization of the HCMV-T_h and T_c Cell Lines and Clones

All of the T_h and T_c lines and clones employed in the Examples hereinbelow were characterized as to phenotype, proliferative responses, IL-2 production and cytotoxic activity as follows:

A. Phenotype Analysis.

T cell lines were analyzed for expression of CD phenotypic determinants using the monoclonal antibodies OKT3 (total T cells), OKT4 (helper/inducer T cells), and OKT8 (cytotoxic/suppressor T cells) (Ortho Pharmaceuticals Inc., Raritan, NJ) using an indirect immunofluorescence assay. Fluorescence was detected either by fluorescence microscopy using a Zeiss flourescent microscope or flow cytometry

using the EPICS sp cell 541 (Coulter Corp., Hialeah, FL). Polyclonal T_h lines were predominantly CD3+4+8-; all T_h clones expressed the CD3+4+8- phenotype. T_c lines were predominantly CD3+4-8+; T_c clones were CD3+4-8+.

B. Lymphocyte Proliferation.

T cell lines and clones were rested in tissue culture media in the absence of TCGF overnight, and then restimulated for 72 hrs with either whole HCMV antigen or HSV antigen to determine the specificity of proliferative response of T_h clones. All T_h lines and clones demonstrated positive proliferative responses to HCMV, whereas responses to HSV were similar to tissue culture media background control. Thus, all T_h lines and clones were HCMV-specific. T_c cell clones proliferated poorly to HCMV antigen in the absence of IL-2.

C. Interleukin-2 (IL-2) Production.

T_h clones were stimulated with whole HCMV antigen, and the supernatants harvested at 24 hrs and assayed for IL-2 activity using the murine CTLL-20 (IL-2 dependent) cell line. All T_h clones were shown to produce IL-2, as demonstrated by the survival and growth of CTLL-20. T_c clones were not tested for IL-2 production.

D. Cytotoxic Activity.

All T_h clones were tested for cytotoxic activity against the NK target cell line, K562; autologous uninfected and CMV- and HSV-infected fibroblasts as target cells. No NK

or virus-specific cytotoxic activity was observed with any of the T_h clones.

All T_c clones were tested for cytotoxic activity against the NK target cell lines, K562; autologous and unmatched or partially matched allogeneic uninfected and HCMV-, HSV-, and influenza-infected fibroblasts as target cells. No anti-NK activity was observed with any of the T_c clones. All HCMV-specific T_c clones exhibited cytotoxic activity against autologous and MHC LA-matched HCMV-infected fibroblasts, but not against allogeneic unmatched HCMV-infected fibroblasts. No cytotoxic activity was observed to any target cells infected with HSV or influenza virus. Thus, the T_c clones described herein exhibit HCMV-specific cytotoxic activity restricted by MHC class I antigens, and therefore exhibit the characteristics of virus-specific cytotoxic T cells.

EXAMPLE III

Proliferative Response of HCMV-Specific T_h Clones to HCMV Antigens

Seventeen HCMV-T_h clones obtained from donor WRC were characterized extensively as to phenotype, proliferative responses, IL-2 production and cytotoxic activity. All clones were CD3+4+8-; proliferative to HCMV but not HSV (as described in Example II (2) above), all produced IL-2 when stimulated with HCMV but not HSV, and none exhibited cytotoxic activity. Thus, all clones were considered to be T helper cells. All clones also exhibited class II MHC restriction specificity, as determined by blocking with anti-class II

monoclonal antibodies and reactivity to whole HCMV antigen presented by autologous or allogeneic antigen presenting cells sharing the appropriate Dw restriction determinant expressed in association with DR, DQ or DP.

For studies of T cell reactivity to purified HCMV antigens, x-irradiated autologous mononuclear cells were added as a source of antigen-presenting cells in a 3-day restimulation culture. One µCi per well of tritiated thymidine was added for the final 16 hours of culture. Cultures were harvested on glass fiber filters and counted in a liquid scintillation spectrophotometer. The results of these assays are summarized on Table 3, below.

TABLE 3

Reactivity of HCMV-Specific T_h Clones to Whole HCMV Antigen, HCMV Detergent Extract (Envelope Glycoproteins), Precipitate from Detergent Extract (Internal Proteins), and HPLC-Purified HCMV Glycoproteins.

	Proliferative Response of HCMV-T_h Clone in Counts per Min. (CPM)							
			Clone + MNC^{IRR} + CMV Antigen					
Clone No.	Clone Alone	Clone + MNC^{IRR}	Whole HCMV Antigen	HCMV Detergent Extract	Towne Capsid Ppt.	HPLC-2UR*	HPLC-3UR*	HPLC-4UR*
WRC-T2#69	98	117	73,282	4,855	6,186	178	142	161
WRC-T3#3	67	1,011	55,080	9,311	3,355	1,194	19,706	25,387
WRC-T3#4	111	475	51,185	15,632	1,580	163	365	155
WRC-T3#16	87	142	121,030	4,553	154	142	308	155
WRC-L6	114	142	63,867	5,669	4,433	213	118	427
WRC-L7	241	1,079	70,484	29,131	7,439	2,338	585	520
WRC-L8	124	150	33,977	10,120	2,742	800	189	204
WRC-L10	325	981	70,773	32,569	10,307	10,321	74,902	31,063
WRC-L14	217	315	68,558	39,805	3,158	389	1,073	277
WRC-L25	399	64	89,847	36,149	7,994	614	678	330
WRC-L43	181	670	102,787	16,722	16,389	404	496	1,495
WRC-T3#8	48	1,834	20,297	33,736	15,641	229	172	299
WRC-T2#41	103	480	6,200	3,880		262	1,346	4,215
WRC-L31	122	268	31,497	45,766	26,927	276	114	206
WRC-L34	52	319	12,477	15,077	8,762	196	187	253
WRC-L35	49	134	5,658	22,965	2,682	148	120	211
WRC-L15	94	131	4,108	1,639		98	163	140

*Purified, unreduced glycopeptide complexes derived from HPLC peaks 2(2UR), 3(3UR) and 4(4UR) as shown on Table 1. HPLC-2UR,4UR contains primarily gpB, reactive with MoAb 9E10; 2UR also contains an immunologically distinct gpC, which is non-reactive with MoAb's reactive with either gpA or gpB. HPLC-3UR contains primarily gpA reactive with MoAb 41C2.

The data summarized on Table 3 demonstrate that all 17 clones were reactive to whole Towne HCMV antigen, displaying from 4108 counts/minute to 121,030 counts/minute. These results suggest that the immunodominant determinant(s) recognized by these HCMV-T_h clones are expressed by structural proteins and/or glycoproteins from the virion.

All clones also reacted significantly to Triton X-100 extracts of Towne HCMV, although to a lesser extent than that to whole Towne viral particles. Thus, it appears that the majority of T_h clones either recognize primarily proteins or glycoproteins included within the envelope of the virus, or proteins that associate sufficiently with the viral envelope to be recovered in the detergent extract. The lower responses compared to whole Towne antigen may reflect inhibitory effects of residual detergent, or a loss of immunogenicity due to structural alterations resulting from the extraction proceoure.

Of interest, all T_h clones also showed a low but significant proliferative response to precipitates of viral antigen following detergent extraction. While it is likely that some residual envelope glycoproteins and tegument proteins are included within the precipitate, it is also possible that nucleocapsid proteins, which presumably comprise the dominant protein in the precipitate, may also express antigenic determinants recognized by T helper cells.

The T cell clones were then stimulated with purified glycoprotein complexes obtained by anion exchange HPLC fractionation of unreduced detergent extract of whole HCMV antigen (see Example I (1)).

As set forth in B. Kari et al., J. Virology, 60, 345 (1986), and in Table 1, hereinabove, glycoprotein complexes contained within peaks 2UR and 4UR

include a glycoprotein complex recognized by a monoclonal antibody (9E10). This antibody is cross-reactive with several other viruses, and is capable of neutralizing HCMV in the absence of complement. In contrast, a separate and distinct group of glycoprotein complexes are isolated to a high degree of purity in peak 3UR, and are recognized by a separate class of monoclonal antibodies which uniquely react with HCMV, and neutralize HCMV efficiently only in the presence of complement.

Following reduction, the principal glycoproteins derived from the 3UR complex have molecular weights of 130,000; 90,000; and 50,000-52,000 kD; the two glycoproteins derived from the 2UR/4UR complex recognized by the 9E10 monoclonal antibody have molecular weights of 93,000 and 50,000-52,000 kD. A third glycoprotein has been demonstrated in peak 2UR which is distinct from those glycoproteins recognized by the 9E10 monoclonal antibody. Therefore, there are at least three major glycoprotein complexes contained within the envelope of HCMV that have been identified as incorporating in excess of 95% of the total glycoproteins within the HCMV viral envelope. Since these HPLC methods appear to yield glycoprotein complexes of >95% purity, it was of interest to demonstrate the pattern of T_h reactivity to these individual glycoprotein complexes.

As shown in Table 3, of the seventeen T_h clones tested for reactivity with HPLC-purified glycoprotein complexes, two responded to peaks 3UR and 4UR but not to peak 2UR. A third T_h clone responded to all three HPLC peaks. It is likely that these clones are primarily responding to the major glycoprotein complex which is also recognized by monoclonal antibodies which detect the glycoprotein complex principally isolated in

peak 3UR. It is likely that tailing of peak 3UR into peak 4UR accounts for the apparent cross-reactivity. This specificity was confirmed for T_h clone WRC-T3#3, WRC-T2#41, and WRC-L10, which proliferated specifically to glycopeptide gpA produced by m-RNA translation of the gpA gene in vitro (data not shown).

Of particular interest, the majority of clones (14/17) do not appear to recognize determinants expressed on any of the major envelope glycoprotein complexes, as represented by materials 2UR, 3UR and 4UR. Thus, the immunodominant HCMV antigenic determinant recognized by these cloned T helper cells may reside either in a nonglycosylated membrane protein or in an internal protein. If so, this may be similar to influenza virus, in which antibody recognition involves primarily the surface glycoproteins (i.e., hemagglutinin), whereas cytotoxic T lympnocytes are known to recognize primarily internal nucleocapsid proteins.

Table 4 summarizes data regarding the specificity of HCMV-specific T_h clones reactive with individual HCMV (glyco)proteins. Clones were generated by initial stimulation of MNCs from seropositive donor WRC with whole HCMV antigen to select for T_h reactive with structural proteins and glycoproteins of HCMV.

TABLE 4

Specificity of HCMV-Specific T_h Clones Reactive with Individual HCMV (Glyco)proteins

	Proliferative Responses						
			Clone + MNC* + Antigen[a]				
WRC Clones[b]	Clone Alone	Clone + MNC*[c]	Whole HCMV Virion	gpA	HPLC-Purified 64 kD Protein	HSV	Adeno
T3 #3	405	805	35,666	19,706	N.D.	683	211
T2 #41	871	746	12,410	6,158	N.D.	584	701
T2 #88	667	162	28,163	112	29,284	173	114
T2 #131	271	288	28,036	N.D.[d]	14,426	135	273
L33	522	901	5,366	188	488	300	791
L42	341	238	13,360	666	317	225	197

a. Proliferative response measured as uptake of ^{3}H-thymidine in CPM; the following antigens were used:
   (i) Whole CMV Virus: Towne strain CMV virus purified from cell culture supernatant on sucrose gradients.
   (ii) Envelope glycoprotein gpA: peak 3UR purified by anion exchange HPLC of detergent extract of Towne CMV.
   (iii) 64 kD matrix protein: partial purification by reverse phase/gel filtration HPLC.
   (iv) HSV, Adeno: whole virus purified from supernatants of infected cell cultures

b. WRC T_h clones obtained by initial stimulation with whole Towne virus; 18% of T_h clones were gpA specific; 33% of T_h clones were specific for HPLC-purified 64 kD protein.

c. MNC* = Irradiated autologous MNC as APC

d. N.D. = Not determined

Eighteen percent of T_h clones tested were specific for gpA as described in detail in Example I.1. Representative examples include WRCT3#3 and WRCT2#41. Thirty-three percent of the HCMV-specific T_h clones were found to be reactive with whole virus as well as a partially-purified matrix protein of molecular weight 64,000 daltons. This protein was obtained by reverse phase HPLC and gel filtration, according to a modification of the method of B. R. Clark, J. Virol., 49, 279 (1984). Whole Towne HCMV obtained by centrifugation of the supernatant of the HCMV-infected fibroblasts was solubilized in 6 M guanidinium chloride and run on reverse phase HPLC with a C-18 column. Proteins adsorbed to the column were eluted with acetonitrile and detected by monitoring the column effluent at 214 nanometers. Peaks collected were pooled and the 64 kD protein was identified by SDS-PAGE.

The 64 kD protein was further purified from co-eluted proteins by size-exclusion chromatography using TSK 4000 and TSK 3000 SEC columns linked in series. An aggregate form of the 64 kD protein was isolated as determined by SDS-PAGE using this method.

Clones WRC-T2/88 and WRC-T2/131 are representative of HCMV-specific T_h clones which proliferated in response to the 64 kD matrix protein. Of interest, many of the clones (i.e., WRC-L33 and WRC-L42) were non-reactive with either the major envelope glycoprotein, gpA, or the abundant 64 kD internal matrix protein. Thus, it is apparent that HCMV-specific T helper cells exist to a variety of structural HCMV proteins.

EXAMPLE IV

Cytotoxic Responses of HCMV-Specific T_c Lines and Clones to HCMV Antigens

Polyclonal and monoclonal T_c cells were assayed for cytotoxicity against the NK target cell,

K562; and autologous or allogeneic fibroblasts infected with HCMV, HSV or influenza virus. The following specific methodologies were used:

A. Preparation of Target Cells

1. HCMV-Infected Human Skin Fibroblasts (SF)

Human diploid skin fibroblasts (SF) were obtained from skin biopsies of adult donors and newborn foreskins. Cells were passaged at least 6 times, and frozen at 1°C/minute in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO) and stored in liquid nitrogen. Before use, cells were thawed in a 37°C water bath, washed with media, and seeded onto 25 cm^2 tissue culture flasks. SF were grown to confluence, infected with HCMV at a multiplicity of infection of 0.2, and incubated until 70-90% of the monolayer demonstrated cytopathic effect. Cells were then labelled with Na_2CrO_4 (375 uCi in 2 ml medium) overnight at 37°C in an atmosphere of 5% CO_2. The cells were washed three times, and resuspended in RPMI in 10% FCS. Uninfected ^{51}Cr-labelled SFs were used as controls.

2. HSV- and Influenza-infected Skin Fibroblasts

An SF monolayer in a 25 cm^2 flask was infected with HSV or influenza at a multiplicity of infection of 5-10 and incubated overnight. The cells were labelled the same way as HCMV-infected SF.

3. K562

The erythroleukemia cell line K562 was used as a source of target cells to measure NK activity. K562 cells (1×10^6) were suspended in 1 ml medium and 375 µCi of Na_2CrO_4 in a 25 cm^2 flask overnight at 37°C in an atmosphere of 5% CO_2). The cells were resuspended in RPMI supplemented with 10% FCS after three washings.

4. SF Which Selectively Express I-E HCMV Proteins

SFs were infected with HCMV at a multiplicity of infection of 5-10 for three hours in the presence of cyclohexamide (15 µg/ml). After removal of cyclohexamide, Actinomycin-D (5 µg/ml) and Na_2CrO_4 (375 µCi) were added to the culture. The cells were then washed three times and resuspended in RPMI in 10% FCS as target cells. The cytotoxicity assay was performed under Actinomycin-D to prevent DNA transcription. Uninfected SFs treated with cyclohexamide and Actinomycin D in parallel were used as control.

B. Cytotoxicity Assay

Three thousand target cells in 100 µl were added to each well of V-bottomed microtiter plates. Serial dilutions of effector cells in 100 µl were then added to the target cells to yield effector-to-target ratios of 50:1, 25:1, and 12:1. The plates were centrifuged at 50 g for 5 minutes at 37°C in an atmosphere of 5% CO_2 for 10 hours. One hundred µl of supernatants were harvested and counted in a gamma counter (Beckman Gamma 9000). Controls consisted of maximum lysis and spontaneous release obtained by

adding 100 µl of Triton-X-100 in medium respectively to target cells. Calculations are done according to the following formula:

$$\%^{51}Cr \text{ release} = \frac{\text{Experimental CPM} - \text{spontaneous release CPM}}{\text{Maximum CPM} - \text{spontaneous release CPM}}$$

Experimental counts per minute represented cells from wells with effector cells.

Specific release (SR) = ($\%^{51}Cr$ release HCMV-infected targets) - ($\%^{51}Cr$ release uninfected targets).

Results were expressed as the mean from triplicate or quadruplicate wells. Spontaneous release from both infected and uninfected SF target cells was less than 40%.

1. Cytotoxic Activity of HCMV-Specific Polyclonal T_c Cell Lines Stimulated with Towne HCMV-Infected Autologous Skin Fibroblasts (SF)

Mononuclear cells from seropositive donor SP-CN were stimulated for 7-10 days in bulk culture with autologous skin fibroblasts (SF) infected for 20 hours with Towne HCMV. T cell blasts were restimulated weekly with HCMV-infected fibroblasts, autologous irradiated MNC as feeder cells and IL-2. Resulting polyclonal CD8+ T cell lines were evaluated for cytotoxic activity against uninfected and HCMV-infected autologous fibroblasts and the NK target cell line K562. The results of this study are presented on Table 5, below.

TABLE 5

Cytotoxic activity of SP-CN cell lines stimulated with Towne HCMV-infected autologous skin fibroblasts (SF)

Effector Cells	E:T ratio[a]	Lysis of target cells[b]				
		Uninfected Autologous SF	HCMV 20 hr.-Infected Autologous SF	CH-act.D. treated Autologous SF	HCMV Infected CH-act.D. Autologous SF	K562
SP-CN CA line 1	50:1	9.1	57.5	11.2	28.9	56.9
	25:1	5.1	44.4	9.7	25.3	46.0
	12:1	5.2	40.8	4.8	23.1	36.4
SP-CN CA line 2	50:1	6.7	40.7	6.6	20.5	31.0
	25:1	4.6	42.4	6.3	19.2	26.4
	12:1	4.3	27.9	-3.7	21.6	8.1

a. Effector:target cell ratio

b. Values expressed as mean specific ^{51}Cr release from quadruplicate wells

As can be seen from the data presented on Table 5, both cell lines exhibited cytolytic activity against autologous HCMV 20 hour-infected fibroblasts expressing immediate-early and late viral proteins; and against CMV-infected autologous fibroblasts that had been treated with cyclohexamide (CH) and Actinomycin D (act. D) to selectively express the immediate-early proteins of HCMV. Both lines also expressed significant levels of NK-like cytotoxic activity.

2. Cytotoxic Activity of HCMV-Specific T_c Cell Lines and Clones Stimulated with Sucrose Gradient-Purified Towne HCMV Viral Particles

CD8+ T_c cell lines and clones were obtained from seropositive donor SP-RK by initial stimulation of MNC with sucrose gradient-purified Towne HCMV viral particles, followed by repeated stimulation with HCMV viral particles, autologous irradiated MNC as feeder cells, and IL-2. Three CD8+ lines and the CD8+ SP-RK clone #3 lysed HCMV-infected autologous fibroblasts but not allogeneic unmatched HCMV-infected fibroblasts or autologous fibroblasts infected with either HSV or influenza virus as shown in Table 6, below.

TABLE 6

Cytotoxic activity of SP-RK T cell lines and clone stimulated with sucrose gradient-purified Towne HCMV viral particles

Effector Cells	E:T ratio	Lysis of target cells: Uninfected SP-RK SF	HCMV 20 hr.-Infected SP-RK SF	HSV infected SP-RK SF	Influenza Virus Infected SP-RK SF	Uninfected MHC Class I Unmatched SF	HCMV 20 hr.-Infected MHC Class I Unmatched SF	K562
SP-RK line 7	50:1	0.6	20.4	-1.6	4.1	6.8	2.5	13.8
	25:1	1.6	19.0	0.0	4.4	8.3	3.0	11.9
	12:1	1.8	15.7	-0.9	0.7	2.5	1.9	11.6
SP-RK line 1	50:1	2.2	34.4	-3.7	5.7	5.6	2.8	11.4
	25:1	2.0	33.1	-0.4	6.7	6.3	4.9	9.0
	12:1	-1.6	28.5	-7.2	4.6	5.1	1.8	6.9
SP-RK line 4	50:1	3.0	21.3	-2.9	7.0	6.3	2.9	18.4
	25:1	3.4	22.3	0.8	7.2	6.3	8.9	18.9
	12:1	2.8	18.6	0.5	0.6	5.1	1.9	19.5
SP-RK clone 3	50:1	1.3	33.4	2.5	5.2	7.5	1.1	17.3
	25:1	2.8	33.0	0.9	7.5	7.7	7.3	18.7
	12:1	2.3	36.6	-0.1	6.5	8.8	11.3	11.0

These lines expressed little cytotoxic activity against the K562 NK target cell. Thus, these T cell lines and clone are characteristic of HCMV-specific, MNC class I LA-restricted T cytotoxic cells which recognize an antigen(s) expressed by whole viral antigen (i.e., structural proteins).

EXAMPLE V

Augmentation of Antigen-Induced Proliferation of HCMV-Specific T_h Clones Using Polyclonal Anti-Serum from Seropositive Donors and HCMV-Specific Monoclonal Antibodies

1. Monoclonal Antibody.

HCMV-T_h clone WRC-T3#3 was used at a concentration of 1.5×10^4 cells per well as a source of responder cells. Autologous x-irradiated MNC (10^5) were added as a source of antigen-presenting cells. An optimal dilution of whole HCMV antigen or 0.1 µg/ml of HPLC-purified 3UR was added as a source of HCMV-specific antigenic stimulation. Serial dilutions of monoclonal antibodies 41C2, which recognizes the immunodominant glycoprotein A (gpA) or a control monoclonal antibody 35F10, which recognizes a nonglycosylated protein designated protein D were added at concentrations ranging from 10 down to 0.01 µg/ml protein per well. The results of this experiment are summarized in Table 7, below.

TABLE 7

MoAb Directed Against HCMV Glycoprotein A (gpA) Augments the Proliferative Response of gpA-Specific T_h Clone WRC-T3#3

	Proliferative Response of WRC-T3#3				
	MoAb	MoAb 41C2 (anti-gpA)		MoAb 35F10 (anti-pD)	
MoAb Concentration (ug protein/well)		3UR	Whole HCMV Ag[b]	3UR	Whole HCMV Ag[b]
10		19,667[a]	5,674	17,577	2,349
3		32,526	8,774	18,195	3,508
1		34,713	12,528	18,160	4,691
0.3		41,996	14,271	16,324	6,371
0.1		36,300	8,143	16,618	4,835
0.03		31,486	9,252	19,435	4,349
0.01		14,406	8,442	17,327	3,676

[a] Counts per minute.

[b] Whole HCMV viral antigen (strain AD169) used at a 1:1000 dilution.

The data summarized in Table 7 demonstrate that monoclonal antibody 41C2 significantly augmented the proliferative response of the T_h clone when 3UR or whole HCMV antigen was used for antigenic stimulation. In contrast, no significant augmentation was observed with the monoclonal antibody directed against protein D using either 3UR or whole HCMV antigen.

Thus, it appears that monoclonal antibodies directed against an immunogenic glycoprotein complex which specifically stimulates a particular T_h clone, is effective to enhance the proliferative response.

Six HCMV-T_h clones shown to be responsive to whole HCMV antigen were then stimulated with whole HCMV

antigen plus serial dilutions of either monoclonal antibody 41C2 (to gpA) or monoclonal antibody 35F10 (to protein D). Seropositive and seronegative whole sera were also tested under the same conditions. The results of these experiments are summarized in Table 8, below.

TABLE 8

MoAb Reactive with HCMV Glycoprotein A (gpA) Augments the Proliferative Response of HCMV-Specific T_h Clones to Whole HCMV Antigen

	Proliferative Response of HCMV-T_h Clones[b]				
Culture Conditions[a]	WRC-T3#8	WRC-T2#41	WRC-T2#69	WRC-L10	WRC-T3#3
Clone alone	167	453	274	121	237
+ MNC^{IRR}	259	273	200	2,995	185
+ MNC^{IRR} and HCMV Ag	19,778	3,076	5,751	8,936	20,734
MoAb 41C2 (anti-gpA)					
10 µg/ml	25,913	6,948	9,565	16,128	25,292
3 µg/ml	26,275	8,627	11,321	17,224	19,370
1 µg/ml	22,073	7,815	9,577	17,183	20,599
0.3 µg/ml	21,558	6,867	8,010	14,807	20,489
0.1 µg/ml	16,677	4,100	5,210	11,456	21,482
0.03 µg/ml	17,026	4,202	6,283	8,063	16,002
0.01 µg/ml	16,315	3,294	5,403	7,670	18,069

MoAb 35F10 (anti-pD)					
10 µg/ml	18,587	6,737	4,683	7,584	18,840
3 µg/ml	19,100	5,061	6,616	9,488	18,967
1 µg/ml	20,471	5,903	6,382	7,376	17,799
0.3 µg/ml	18,916	8,001	4,826	8,342	18,605
0.1 µg/ml	18,842	3,542	5,404	7,570	18,518
0.03 µg/ml	17,439	3,550	6,321	9,226	18,993
0.01 µg/ml	18,248	2,964	5,971	7,262	22,511
Seropositive Whole Serum (Donor CR)					
3.3 lambda/ml	51,068	11,412	17,616	16,345	24,918
Seronegative Whole Serum (Donor SF)					
3.3 lambda/ml	27,506	6,784	8,103	15,464	20,445

[a] $1.5X10^4$ T_h/well + 10^5 autologous MNC^{IRR}/well + whole HCMV antigen.

[b] Counts per minute.

Surprisingly, monoclonal antibody 41C2, directed against gpA, augmented the proliferative response of all six T_h clones to whole HCMV antigen, despite the fact that only 3 of the 6 clones respond to HPLC-purified gpA in the absence of antibody (see Table 7). The monoclonal antibody directed against protein D did not augment the response of any of the clones tested. Whole serum from the seropositive donor augmented the response of all clones, whereas that from

the seronegative donor did not. These data suggest that the mechanism of augmentation does not necessarily involve a direct interaction between the antibody and the specific viral protein or glycoprotein recognized by the T_h clone.

EXAMPLE VI

Presentation of HCMV Antigen to T Helper Cell Clones by MNC, LCL or Mixtures Thereof

1. Epstein-Barr Virus (EBV)-transformed Lymphoblastoid Cell Lines (LCL).

An autologous LCL from seropositive donor SP-CN (SP-CN LCL) was established by the method of Sugden and Mark, J. Virology, 23, 503 (1977) and grown in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS). AMG LCL (DR 2,2/Dw 2,2) and NHS LCL (DR 3,8) were gifts from Dr. F. Bach (IRC, University of Minnesota, Minneapolis, MN).

2. Generation of HCMV-Specific T Cell Clones.

HCMV-specific T cell clones were generated from a seropositive donor (SP-CN). Briefly, mononuclear cells (MNC) were cultured at 10^6 cells/ml in RPMI 1640 medium supplemented with 15% heat-inactivated HCMV-seronegative human serum (PHS) and 1 µg/ml whole HCMV antigen. After 7-10 days, T cell blasts were isolated and cloned by limiting dilution at 0.3 cells/well in round-bottom wells containing 0.2 ml RPMI-15% PHS medium with 10,000 autologous x-irradiated (5,000 R) MNC, 1 µg/ml whole HCMV antigen (Towne strain) and 15% IL-2 (TCGF, Biotest, Frankfurt, West Germany). Plating efficiencies of 2% to 12% were observed. The clones were expanded in culture medium containing 10-20% IL-2, and

restimulated with autologous x-irradiated MNC/LCL mixture and 1 µg/ml whole HCMV antigen every 1-2 weeks.

3. Proliferation Assay.

HCMV-specific cloned T_h cells were assayed 7-14 days after restimulation. The microcultures were set up in round-bottom plates (Flow Lab., Inc., VA) with a total volume of 0.2 ml/well containing 10^4 HCMV-specific T_h cells, various concentrations of x-irradiated autologous MNC and/or LCL and whole HCMV antigen (Towne strain) or HSV antigen. The cultures were incubated for 3 days, and 1 µCi/well ^{3}H-thymidine (New England Nuclear, Boston, MA) was added to each well for the final 16-24 hours of culture. Wells were harvested with an automatic cell harvester, cells collected on glass fiber filter paper, and radioactivity measured in a Beckman 5801 liquid scintillation spectrometer.

4. LCL Presents HCMV Antigen to HCMV-Specific T_h Clones.

A panel of HCMV-specific T_h clones generated from a seropositive donor (SP-CN) were tested for HCMV-specific proliferative activity using either x-irradiated autologous MNC (10^5/well) or x-irradiated autologous LCL (10^4 - 3 x 10^4/well) as APC. HSV antigen was included as an antigen specificity control. Table 9 shows the results obtained using seven clones.

TABLE 9: PROLIFERATIVE ACTIVITY OF HCMV-SPECIFIC T_h CELL CLONES USING EITHER IRRADIATED AUTOLOGOUS MNC OR LCL

	T_h CELLS + MNC^{IRR}[a] (CPM)			T_h CELLS + LCL^{IRR}[b] (CPM)		
T_h Clone	---	+TOWNE AG[c]	+HSV[c]	---	+TOWNE AG	+HSV
SP-CN/T3-#9	65 ± 6[d]	8,114 ± 2,045	ND	379 ± 105	21,370 ± 3,504	306 ± 148
SP-CN/T2-#41	182 ± 79	9,377 ± 671	154 ± 85	154 ± 32	1,458 ± 379	ND
SP-CN/T2-#34	763 ± 250	10,634 ± 506	403 ± 31	205 ± 158	4,749 ± 156	ND
SP-CN/T3-#16	132 ± 44	10,296 ± 1,468	244 ± 136	206 ± 60	16,738 ± 1,656	413 ± 84
SP-CN/T3-#8	61 ± 7	33,507 ± 2,342	252 ± 343	150 ± 3	1,434 ± 245	141 ± 49
SP-CN/T5-#43	82 ± 20	35,384 ± 4,301	502 ± 230	ND	1,020 ± 104	449 ± 67
SP-CN/T2-#131	278 ± 367	68,307 ± 3,562	78 ± 7	119 ± 29	1,529 ± 216	ND

[a] x-irradiated autologous MNC (5,000 rads) were added at 10^5 MNC/well.

[b] x-irradiated autologous MNC (8,000 rads) were added at 10^4 $3x10^4$/well.

[c] Heat-inactivated (56°C/1 hr.) whole HCMV antigen (Towne) or HSV antigen was added at 2 µg antigen/well.

[d] The data is expressed as (mean ± 1 S.D.) CPM from triplicate wells.

All of the T_h clones listed on Table 9 proliferated well in response to HCMV antigen but not HSV antigen when MNC were added to the cultures as APC. The magnitude of proliferation ranged from 8114 to 68,307 cpm. Most of these clones also exhibited HCMV antigen-specific proliferative responses when LCL were used as APC with a range of 1,020 to 21,370. Clones SP-CN/T2-131 and SP-CN/T5-43 proliferated well to HCMV in the presence of irradiated autologous MNC, but poorly in the presence of irradiated autologous LCL.

On the other hand, clones SP-CN/T3-16 and SP-CN/T3-9 proliferated well to HCMV in the presence of irradiated autologous LCL, although their proliferation to HCMV in the presence of irradiated MNC was no better than the other five clones. Thus, there is no apparent correlation between proliferative response to HCMV using MNC as APC and that using LCL as APC.

It is well known that antigen-specific T helper (T_h) cells recognize antigen associated with class II MHC products on the surface of APC (P. Erb et al., J. Exper. Med., 142, 460 (1975)). We studied the MHC restriction of some of these T_h clones using LCL as APC, and found all of the clones tested are class II restricted. LCL from other MHC class II-matched donors also presented HCMV antigen well to the two high-responding clones (SP-CN/T3-16 and SP-CN/T3-9), but poorly to the five low-responding clones. For example, SP-CN LCL (autologous, DR2,4/DW2,4) as well as AMG LCL (DR2,2/DW2,2) presented HCMV antigen to clone SP-CN/T3-9, whereas NHB LCL (DR3,8) did not present HCMV to the same clone.

5. Augmenting Effect of LCL on Proliferative Responses of T_h Cells to MNC and HCMV.

Facing limited supplies of autologous MNC as APC to stimulate HCMV-specific T_h cells and inefficient antigen presentation by autologous LCL when used alone, combinations of MNC and LCL were employed as APC and feeder cells during reactivation of the T_h clones. Surprisingly, the combination of LCL and MNC showed a synergestic effect on proliferative response as illustrated in Table 10, below.

TABLE 10

PROLIFERATIVE RESPONSES OF T HELPER CELL CLONE SP-CN/T5-43 USING AUTOLOGOUS WRC MNC AND/OR LCL AS APC

ANTIGEN PRESENTING CELLS (CELLS/WELL)		^{3}H-THYMIDINE INCORPORATED (ΔCPM)[a]
MNC^{IRR}	LCL^{IRR}	
---	---	73 ± 34
---	10^4	101 ± 186
---	$3x10^4$	-106 ± 43
---	10^5	179 ± 117
10^4	---	6,048 ± 33
10^4	10^4	13,280 ± 4,019
10^4	$3x10^4$	8,923 ± 952
10^4	10^5	5,089 ± 1,293
$3x10^4$	---	21,541 ± 895
$3x10^4$	10^4	43,336 ± 10,489
$3x10^4$	$3x10^4$	33,730 ± 4,593
$3x10^4$	10^5	16,732 ± 1,304
10^5	---	58,890 ± 4,121
10^5	10^4	77,493 ± 8,728
10^5	$3x10^4$	71,544 ± 1,938
10^5	10^5	47,641 ± 2,313

[a] The proliferation assays were set up by using 10^4 T cells/well, 10^4-10^5 MNC^{IRR} and/or LCL^{IRR}/well and 2µg/well whole Towne HCMV antigen. ^{3}H-thymidine incorporation of clone SP-CN/T5-43 plus SP-CN LCL^{IRR} (10^4-10^5/well) was in the range of 59-92.

^{3}H-thymidine incorporation of clone SP-CN/T5-43 plus SP-CN LCL^{IRR} at LCL concentrations of 10^4/well, $3x10^4$/well and 10^5/well were 629; 1,279; and 2,466; respectively.

LCL alone as APC at a range of 10^4 LCL/well to 10^5 LCL/well did not stimulate any significant T cell proliferative response to whole HCMV antigen above background. However, the addition of LCL at 10^4 LCL/well and 3 x 10^4 LCL/well did augment the 3-day proliferative response of T_h cells to MNC plus whole HCMV substantially, although 10^5 LCL/well seemed to suppress the proliferative responses, probably due to excessive cell density in the culture.

The same cultures were expanded in the presence of 15% IL-2 for 12 days and cell counts were performed on day 7 and day 12 after the beginning of the culture. The results of these experiments are summarized in Table II, below.

TABLE 11

NUMBER OF SP-CN/T5-43 CELLS EXPANDED AFTER RESTIMULATION WITH HCMV ANTIGEN IN THE PRESENCE OF AUTOLOGOUS MNC AND/OR LCL AS APC[a]

ANTIGEN PRESENTING CELLS (CELLS/WELL)		T_h CELLS RECOVERED			
		NUMBER OF CELLS RECOVERED ($\times 10^5$)		RATIO OF T_h CELLS RECOVERED: T_h CELLS STARTED	
MNC IRR	LCL IRR	DAY 7	DAY 12	DAY 7	DAY 12
---	---	<0.1	<0.1	---	---
---	10^4	<0.1	<0.1	---	---
---	3×10^4	<0.1	<0.1	---	---
---	10^5	<0.1	<0.1	---	---
10^4	---	<0.1	0.8	---	3
10^4	10^4	0.4	8.8	1	29
10^4	3×10^4	0.8	18.4	3	61
10^4	10^5	2	6.4	7	21
3×10^4	---	<0.1	1.6	---	5
3×10^4	10^4	7.2	46	24	153
3×10^4	3×10^4	3.2	79	11	263
3×10^4	10^5	2.4	20	8	67
10^5	---	2.4	12.8	8	43
10^5	10^4	8	186	27	620
10^5	3×10^4	10.4	NA[b]	35	NA
10^5	10^5	8	78	27	260

[a] The cultures were set up in parallel with those of Table 6. Cells from triplicate wells under the same conditions were combined on day 3 and expanded in the presence of IL-2 for 12 days.

[b] Not analyzed.

As shown in Table 11, when no MNC were present, T_h cells were not expanded even when both LCL and whole HCMV antigen were added to the culture. A combination MNC and whole HCMV antigen led to a 3- to 43-fold increase in T cell numbers during 12 days of culture depending on the amount of MNC that were involved. When LCL were added along with MNC and HCMV antigen, a 29- to 620-fold increase in T cell numbers was observed over the same period of time under the same tissue culture conditions, clearly indicating that LCL were augmenting the expansion of activated T_h cells.

Aliquots of these cultures were plated in 96-well flat-bottom microtiter plates on day 7 at 0.2 ml/well in triplicate. After overnight pulsing with $[^3H]$-thymidine, a similar augmenting effect of LCL on cell proliferation as measured by incorporated $[^3H]$-thymidine was observed as summarized on Table 12, below.

TABLE 12

[^{3}H]-THYMIDINE INCORPORATION OF DAY 7 CULTURES AFTER RESTIMULATION WITH HCMV ANTIGEN IN THE PRESENCE OF AUTOLOGOUS MNC AND/OR LCL AS APC[a]

ANTIGEN PRESENTING CELLS (CELLS/WELL)		[^{3}H]-THYMIDINE INCORPORATED (CPM)[a]
MNC^{IRR}	LCL^{IRR}	
---	---	134 ± 21
---	10^4	423 ± 440
---	$3x10^4$	599 ± 262
---	10^5	837 ± 170
10^4	---	1,080 ± 72
10^4	10^4	6,908 ± 786
10^4	$3x10^4$	8,726 ± 1,055
10^4	10^5	3,606 ± 143
$3x10^4$	---	3,456 ± 122
$3x10^4$	10^4	27,063 ± 3,488
$3x10^4$	$3x10^4$	24,998 ± 1,610
$3x10^4$	10^5	13,483 ± 355
10^5	---	22,369 ± 1,590
10^5	10^4	58,315 ± 2,159
10^5	$3x10^4$	52,110 ± 3,232
10^5	10^5	37,876 ± 3,533

[a] The cells were taken from cultures shown in Table 7 on day 7 and added to flat-bottomed wells at 0.2 ml/well in triplicate. The cultures were labeled with [^{3}H]-thymidine for 18 hrs. before harvesting.

These results confirmed the feasibility of using both MNC and LCL as feeder cells along with antigen in activation and expansion of T_h clones. The extent of T_h cell expansion is proportional to the concentration of MNC^{IRR} in the range of 10^4 to 10^5 MNC/well, but not proportional to the concentration of LCL^{IRR}. The optimal ratios of LCL to cloned T_h cells are in the range of about 1:1 to 3:1.

Samples of antigen-presenting LCL cell lines SP-CN/T3-43 (Access Code: IVI-10122); and SP-CN/T5-43 (Access Code: IVI-10123) have also been deposited with In Vitro International, Linthicum, MD, in accord with the Draft PTO Deposit Policy for Biological Materials, BNA PTCJ, 32, 90 (1986).

Cultures of these deposited cell lines will be made available to the public upon the grant of a patent based upon the present application. It is to be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by the United States Government.

While certain representations embodied are described herein for the purposes of illustration, it will be apparent to those skilled in the art that modifications therein may be made without departing from the spirit and scope of the invention.

We wish to thank Children's Hospital, St. Paul, Minnesota, for generous support and encouragement in this work.

WHAT IS CLAIMED IS:

1. A method for production of a homogeneous population of T helper cells which are specific for a viral antigen comprising the steps of:

(a) isolating a population of mononuclear cells (MNC) from the blood of a donor mammal, wherein the MNC population comprises T helper cells specific for said viral antigen, and autologous, non-proliferative, antigen-presenting cells (APC);

(b) combining said isolated population of said MNCs with an amount of said viral antigen effective to cause the proliferation of a T helper cell which is specific for said antigen;

(c) allowing said T helper cell to proliferate for a period of time effective to allow non-proliferating MNCs to lose viability; and

(d) clonally-expanding said antigen-specific T helper cell in the presence of an amount of non-proliferative antigen-presenting cells comprising a mixture of (i) autologous MNCs, allogeneic MNCs or mixtures thereof; and (ii) autologous lymphoblastoid cells (LCLs), allogeneic LCLs or mixtures thereof; and an amount of said viral antigen effective to proliferate said cloned antigen-specific T

helper cell, to yield said homogeneous population, and wherein said LCLs are present in an amount effective to cooperate with the MNCs to increase the proliferation rate of the T helper cell.

2. The method of claim 1 wherein additional amounts of said viral antigen and said autologous, non-proliferative APCs effective to cause the proliferation of a T helper cell are added during the course of step (c) to cause further proliferation of said T helper cell.

3. The method of claim 1 wherein, in step (d) the non-proliferative APCs comprise a mixture of (i) autologous MNCs; and (ii) autologous LCLs, effective to proliferate said antigen-specific T helper cell, wherein said LCLs are present in an amount effective to cooperate with the MNCs to increase the proliferation rate of the T helper cell.

4. The method of claim 1 wherein the LCLs are derived from a virus-transformed lymphoblastoid cell line.

5. The method of claim 4 wherein the virus-transformed lymphoblastoid cell line is Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell line.

6. The method of claim 1 wherein said viral antigen is a human cytomegalovirus (HCMV) antigen.

7. The method of claim 6 wherein said HCMV antigen is a viral structural protein.

8. The method of claim 7 wherein the HCMV antigen is present in a viral envelope fraction.

9. The method of claim 7 wherein the HCMV antigen comprises a glycopeptide complex present on the viral surface, or a viral glycopeptide derived therefrom.

10. The method of claim 9 wherein the HCMV antigen is an immunogenic glycopeptide of about 93 KD or an immunogenic glycopeptide of about 50-52 KD, which glycopeptides are present on the viral surface.

11. The method of claim 6 wherein the HCMV antigen is an about 64 kD matrix protein.

12. The method of claim 1 wherein step (b), (c) or (d) is carried out in the presence of an amount of a monoclonal antibody which is effective to increase the rate of proliferation of said T helper cell, wherein the monoclonal antibody is specific for said viral antigen.

13. The method of claim 12 wherein the monoclonal antibody is specific for a human cytomegalovirus (HCMV) antigen.

14. The method of claim 13 wherein the monoclonal antibody is specific for an immunogenic glycopeptide of a molecular weight of about 50-52

kD or an immunogenic glycopeptide of a molecular weight of about 93 kD present on the viral surface.

15. The method of claim 14 wherein the monoclonal antibody is produced by hybridoma IVI-10117, IVI-10118 or IVI-10119.

16. The method of claim 1 wherein step (b), (c) or (d) is carried out in the presence of an effective amount of interleukin-2 (IL-2).

17. A method for production of a homogeneous population of T helper cells which are specific for a viral antigen comprising the steps of:

(a) isolating a population of mononuclear cells (MNCs) from the blood of a donor mammal, wherein the MNC population comprises T helper cells specific for said viral antigen and autologous, non-proliferative antigen-presenting cells (APC);

(b) combining said isolated population of said MNCs with an amount of said viral antigen, and an amount of a monoclonal antibody which is specific for said viral antigen, effective to cause the proliferation of a T helper cell which is specific for said antigen;

(c) allowing said T helper cell to proliferate for a period of time effective to allow non-proliferating MNCs to lose viability; and

(d) clonally-expanding said antigen-specific T helper cell in the presence of an amount of non-proliferative autologous antigen-presenting cells, non-proliferative, allogeneic antigen-presenting cells or mixtures thereof; an amount of said viral antigen, and an amount of a monoclonal antibody which is specific for said viral antigen, said amounts being effective to proliferate said cloned antigen-specific T helper cell, to yield said homogeneous population.

18. The method of claim 17 wherein the viral antigen is an HCMV antigen.

19. The method of claim 17 wherein, in step (d), the autologous non-proliferative APCs comprise non-proliferative MNCs.

20. The method of claim 19 wherein, in step (d), the autologous APCs or the allogeneic APCs further comprise non-proliferative LCLs in an amount effective to increase the proliferation rate of the T helper cell over that caused by the nonproliferative MNCs.

21. A method for production of a homogeneous population of T helper cells which are specific for an antigen present on a human cytomegalovirus (HCMV) structural protein comprising the steps of:

(a) isolating a population of mononuclear cells (MNC) from the blood of a donor mammal, wherein the MNC population comprises T helper cells that have specificity for said human cytomegalovirus (HCMV) antigen and nonproliferating, antigen-presenting cells (APC);

(b) combining said isolated MNC population with an amount of said human cytomegalovirus (HCMV) antigen effective to cause the proliferation of a T helper cell which is specific for said human cytomegalovirus (HCMV) antigen;

(c) allowing said T helper cell to proliferate for a period of time effective to allow non-proliferating MNCs to lose viability; and

(d) clonally-expanding said antigen-specific T helper cell in the presence of an effective proliferating amount of said HCMV antigen, a monoclonal antibody specific for said human cytomegalovirus (HCMV) antigen, and an autologous, antigen-presenting cell selected from the group consisting of x-irradiated autologous MNCs, an x-irradiated Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell line (LCL) and mixtures thereof, to yield said homogeneous population.

22. The method of claim 21 wherein additional amounts of said viral antigen, said monoclonal antibody which is specific for said viral

antigen, and said autologous, non-proliferative APCs, effective to cause the proliferation of said T helper cell, are added during the course of step (c) to cause further proliferation of said T helper cell.

23. The method of claim 21 wherein step (b), (c) or (d) is carried out in the presence of an effective amount of interleukin-2 (IL-2).

24. A method for proliferating a homogeneous population of T helper cells which are specific for a viral antigen comprising combining said homogeneous population of T helper cells with an amount of said viral antigen and an amount of a mixture of non-proliferative, antigen-presenting cells (APC) effective to cause the proliferation of said population of T helper cells, wherein said mixture of APCs comprise (i) autologous or allogeneic virus-transformed lymphoblastoid cells (LCL) and (ii) autologous or allogeneic mononuclear cells (MNC), wherein the LCLs are present in an amount effective to cooperate with the MNCs to increase the proliferation rate of the T helper cells.

25. The method of claim 24 wherein the ratio of LCLs to MNCs is at least about 1:1-10.

26. The method of claim 25 wherein said viral antigen is a human cytomegalovirus (HCMV) antigen.

27. The method of claim 24 further comprising combining said population of T helper cells with

an amount of a monoclonal antibody specific for said antigen, which is effective to increase the proliferation rate of said population of T helper cells.

28. The method of claim 24 further comprising combining said population of T helper cells with an amount of interleukin-2 (IL-2) effective to increase the proliferation rate of said population of T helper cells.

29. The method of claim 24 wherein the ratio of T helper cells to LCLs is about 1:1-3.

30. A method for production of a homogeneous population of T cytotoxic cells which are specific for a human cytomegalovirus (HCMV) antigen comprising the steps of:

(a) isolating a population of mononuclear cells (MNC) from the blood of a donor mammal, wherein the MNC population comprises T cytotoxic cells specific for HCMV viral antigens;

(b) combining said isolated population of said MNCs with (i) an amount of autologous, non-proliferative, antigen-presenting cells (APCs), (ii) an amount of HCMV-infected autologous fibroblasts or whole HCMV antigen, and (iii) an amount of interleukin-2 (IL-2), effective to cause the proliferation of a T cytotoxic cell which is specific for an HCMV antigen expressed by said APCs;

(c) allowing said T helper cell to proliferate for a period of time effective to allow non-proliferating MNCs to lose viability; and

(d) clonally-expanding said antigen-specific T cytotoxic cell in the presence of (i) an amount of HCMV-infected, non-proliferative, autologous antigen-presenting cells, HCMV-infected non-proliferative, allogeneic antigen-presenting cells or mixtures thereof, (ii) an amount of IL-2 and (iii) an amount of autologous HCMV-infected fibroblasts or whole HCMV viral antigen effective to proliferate said cloned antigen-specific T cytotoxic cell, to yield said homogeneous population.

31. The method of claim 30 wherein said donor mammal is a human.

32. The method of claim 30 wherein additional amounts of said viral antigen, said IL-2 and said autologous, non-proliferative APCs effective to cause the proliferation of said T cytotoxic cell are added during the course of step (c) to cause further proliferation of said T cytotoxic cell.

33. The method of claim 31 wherein in step (b) the population of MNCs is combined with HCMV-infected autologous fibroblasts.

34. The method of claim 33 wherein in step (d), said antigen-presenting cells further comprise

non-proliferative MNCs derived from a continuous autologous MNC line in an amount effective to increase the proliferation rate of the T cytotoxic cells in cooperation with the autologous mononuclear antigen-presenting cells.

35. The method of claim 34 wherein the MNC line comprises a virus-transformed lymphoblastoid cell line.

36. The method of claim 35 wherein the virus-transformed lymphoblastoid cell line comprises an Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell line.

37. The method of claim 30 wherein step (b), (c) or (d) is carried out in the presence of an amount of a monoclonal antibody which is effective to increase the rate of proliferation of said T cytotoxic cell, wherein the monoclonal antibody is specific for an HCMV antigen.

38. The method for proliferating a homogeneous population of T cytotoxic cells which are specific for an antigen associated with a pathogen comprising combining said homogeneous population of T cytotoxic cells with an amount of interleukin-2 (IL-2) and an amount of a mixture of non-proliferative, antigen-presenting cells (APC) effective to cause the proliferation of said population of T cytotoxic cells, wherein said mixture of APCs

comprise allogeneic virus-transformed lymphoblastoid cells (LCL) and allogeneic mononuclear cells (MNC) which express said antigen.

39. The method of claim 38 wherein said antigen is a viral antigen.

40. The method of claim 39 wherein said viral antigen is a human cytomegalovirus (HCMV) antigen.

41. The method of claim 38 further comprising combining said population of T cytotoxic cells with an amount of monoclonal antibody specific for said antigen, effective to increase the proliferation rate of said population of cells.

42. A pharmaceutical unit dosage form comprising an amount of a homogeneous population of antigen-specific T lymphocytes comprising T_h cells or T_c cells effective to increase an immune response of a mammalian recipient to a pathological target antigen upon parenteral administration of said dosage form; wherein said population of T lymphocytes is MHC LA-matched with respect to the mammalian recipient; and wherein said T lymphocytes are antigen-specific for said pathological target antigen.

43. A pharmaceutical unit dosage form comprising a mixture of a plurality of homogeneous populations of antigen-specific T lymphocytes;

wherein said mixture comprises T_h cells, T_c cells or mixtures thereof; wherein each of said populations is specific for a pathological target antigen; wherein at least one of said plurality of T lymphocyte populations is MHC LA-matched with respect to a mammalian recipient; and wherein said mixture is effective to increase an immune response of the mammalian recipient to at least one pathological target antigen upon parenteral administration of said dosage form.

44. A pharmaceutical unit dosage form comprising a plurality of separately packaged homogeneous populations of antigen-specific T lymphocytes comprising T_h cells or T_c cells; wherein each population comprises T lymphocytes specific for a pathological target antigen; wherein each population is effective to increase an immune response of an MHC-matched mammalian recipient to said pathological target antigen upon parenteral administration of said population of T lymphocytes; and wherein at least one of said populations is MHC LA-matched with respect to the mammalian recipient.

45. A pharmaceutical unit dosage form comprising an amount of a heterogeneous population of antigen-specific T lymphocytes comprising T_h cells, T_c cells or mixtures thereof effective to increase an immune response of a mammalian recipient toıat least one pathological target antigen upon parenteral administration of said population of T lymphocytes; and wherein at least a portion of said population of T lymphocytes are MHC LA-matched with respect to the mammalian recipient.

46. A pharmaceutical unit dosage form prepared by a process comprising combining a plurality of homogeneous populations of antigen-specific T lymphocytes comprising T_h cells or T_c cells with a pharmaceutically-acceptable liquid carrier; wherein each T lymphocyte population is antigen-specific for a pathological target antigen; and wherein at least one T lymphocyte population is MHC LA-matched with respect to the mammalian recipient.

47. The pharmaceutical unit dosage form of claims 44, 45 or 46 wherein said plurality of T lymphocyte populations comprise antigen specificities for a plurality of antigens associated with a particular pathological target.

48. The pharmaceutical unit dosage form of claims 44, 45 or 46 wherein said plurality of T lymphocyte populations comprise antigen specificities for a plurality of HCMV-associated antigens.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/00383

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 5/00; A 61 K 35/14

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	The Journal of Immunology, volume 129, no. 3, September 1982, The American Association of Immunologists, (US), J.R. Lamb et al.: "Antigen-specific human T lymphocyte clones: mechanisms of inhibition of proliferative responses by xenoantiserum to human nonpolymorphic HLA-DR antigens", pages 1085-1090 see the whole article --	1
Y	Biological Abstracts, volume 80, no. 4, 1985, (Philadelphia, PA., US), L.E. Wallace et al.: "Allospecific T cell recognition of HLA-A2 antigens: Evidence for group-specific and subgroup-specific epitopes", see abstract 31796, & Immunogenetics 21(3): 201-214, 1985 --	1
Y	Biological Abstracts, volume 84, no. 10, 1987, (Philadelphia, PA., US), ./.	1

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27th June 1988	18 JUIL. 1988
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	Y.-N. Liu et al.: "Epstein-Barr virus-transformed lymphoblastoid cell lines as antigen-presenting cells and "augmenting" cells for human CMV-specific helper clones", see page AB-452, abstract 98132, & Cell Immunol. 108(1): 64-75, 1987 --	
A	Biological Abstracts, volume 66, 1 November 1978, (Philadelphia, PA., US), J.A. Zaia et al.: "Specificity of the blastogenic response of human mononuclear cells to herpesvirus antigens", see abstract 52179, & Infect. Immun. 20(3): 646-651, 1978	
